Kinetics of valinomycin-mediated K⁺ ion transport through tethered bilayer lipid membranes

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Abstract

Bilayer lipid membranes tethered to planar gold electrodes were prepared, based on a self assembled monolayer (SAM) of 2,3-di-O-phytanyl-sn-glycerol-1-tetraethylene glycol-DL-α-lipoic acid ester lipid (DPTL). When the SAM’s were exposed to a suspension of liposomes made from diphytanoylphosphatidyl choline (DPhyPC), tethered lipid bilayers (tBLMs) were formed with good sealing properties. The preformed tBLMs were doped with valinomycin, and the K⁺ ion concentration in the bathing solution was increased stepwise by adding KCl. Electrical impedance spectra were recorded following every addition. These data were modelled by means of the network simulation program SPICE, using parameter values of the undoped membrane and a kinetic scheme for the K⁺/valinomycin system, whose rate constants were determined previously in independent measurements. Experimental and simulated data are in reasonable agreement despite some simplifying assumptions used in the SPICE simulations. Experimental data were also fitted to a conventional equivalent circuit, which reveals that the representation of the K⁺/valinomycin system by an ohmic resistor can be considered as no more than an approximation.

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1. Introduction

Bilayer lipid membranes tethered to a metal electrode (tBLMs) have been designed as model systems of biological membranes (for recent reviews see, e.g. Refs. [1–3]). tBLMs are considered as a promising tool for the electrochemical investigation of membrane proteins in a quasi-natural environment [4,5]. They are formed from so-called thiolipids, i.e. lipid molecules attached to a metal such as gold or mercury by a terminal sulfur group, and separated from the support by a hydrophilic spacer. Spacer or tether molecules, mostly polyethylene glycol [6,7] or oligopeptides [8,9] are designed to accommodate an aqueous subphase thus mimicking the cytosol.

Valinomycin in the presence of K⁺ ions has been used as a test system for electrochemical impedance spectroscopy (EIS) studies on tBLMs [9,10]. Data thus obtained are usually analyzed by fitting to an equivalent circuit, in which the flow of K⁺ ions through the membrane is represented by a resistor. However, this could be an oversimplification since the valinomycin-mediated K⁺ transport is unlikely to show ohmic behavior even for small amplitudes of the a.c. signal. The limitation can be overcome by using the simulation program SPICE which not only allows one to simulate electrical networks but also bioelectrochemical processes involving membranes [11]. In order to demonstrate the feasibility of this approach a valinomycin doped tBLM is exposed to different K⁺ concentrations, and electrochemical impedance spectra are recorded. These spectra are then simulated with SPICE using parameter values for the K⁺/valinomycin system which were previously determined from independent experiments [12].

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2. Experimental

The thiolipid 2,3-di-O-phytanyl-sn-glycerol-1-tetra-ethylene glycol-DL-ζ-lipoic acid ester lipid (DPTL) was synthesized as described [13]. Diphtanylyphospho- datidyld choline (DPhyPC) (Avanti Polar Lipids, Inc., Alabaster, AL) was used for the preparation of liposomes. Ultrapure water with > 18 MΩ cm and TOC < 3 ppb was from a MilliQ System Synthesis A10. Valinomycin, KCl (both Sigma), and NaCl (SigmaUltra) were used without purification.

2.1. Preparation of template stripped gold substrates

Gold films of 60 nm thickness were deposited by electrothermal evaporation (rate 0.01–0.05 nm/s, 2 × 10⁻⁶ mbar, in a Balzers evaporation system) on freshly cleaved mica sheets. The gold surface was annealed by heating to 650°C for 45 s to form Au(111). The gold surface was then glued with EJOTEK 377 (Polytec GmbH, Waldbronn, Germany) to high refractive index (n = 1.7) glass slides and heated for 60 min to 150 °C. After cooling down, the gold films on glass were separated from the mica sheets and thoroughly rinsed with ethanol.

2.2. Formation of self assembled monolayers of the thiolipid and vesicle fusion

Template stripped gold (TSG) slides were placed for 24 h in an ethanolic solution of 0.2 mg/ml of DPTL, rinsed in pure ethanol and dried in a stream of nitrogen. Liposome stock solutions were prepared by extrusion of a suspension of 2 mg/ml DPhyPC in 0.1 M NaCl solution through a polycarbonate filter with 50 nm nominal pore size. The slides were mounted into the measuring cell and soaked in NaCl solution for at least 14 h. Liposomes were then added from the stock solution to a final concentration of 0.02 mg/ml, and fusion with the DPTL monolayer was allowed to proceed at 32 °C for about 15 h. After rinsing with NaCl solution to remove excess liposomes, valinomycin was added from an ethanolic stock solution to a final concentration of 4 × 10⁻⁶ M.

2.3. Simultaneous surface plasmon resonance and electrochemical impedance spectroscopy

TSG slides were clamped into the measuring cell of a setup described earlier [14] to perform surface plasmon resonance (SPR) and EIS experiments simultaneously. The thickness of the self assembled monolayer (SAM) was measured by SPR, and electrochemical impedance spectra were taken in NaCl solution (0.1 M) before and after the addition of DPhyPC vesicles. Surface plasmons were excited by a 632.5 nm HeNe laser. EIS measurements were conducted using an EG&G potentiostat 273 and a FRA 1260 from Solartron. Spectra were recorded over a frequency range of 1 MHz–10 mHz with an excitation amplitude of 10 mV and a bias potential of 0 V vs. a Ag/AgCl | NaCl(sat) reference electrode, using a platinum counter electrode. Fitting of spectra to an equivalent circuit was performed with the program zview (version 2.6, Scribner Associates, Inc.).

3. Results

3.1. Formation and characterization of the tBLM

tBLMs are generally based on so-called thiolipids, which are amphiphilic supermolecules composed of a hydrophilic spacer terminated by a sulphur linker functionality and a hydrophobic lipid tail. These molecules interact with metal surfaces, e.g. gold, thus forming SAMs hydrophobic to the outside. Once exposed to a suspension of liposomes, they tend to fuse, thus forming lipid bilayers tethered to the metal by the hydrophilic spacer. The thiolipid 2,3-di-O-phytanylsn-glycerol-1-tetraethylene glycol-DL-ζ-lipoic acid ester lipid (DPTL) used in the present investigation has been introduced only recently [13]. As illustrated in Fig. 1A, it consists of two phytanyl lipid groups attached to a glycerol-tetraethyleneoxy spacer. Both kinds of functionalities are held responsible for the excellent sealing properties of tBLMs prepared from this compound. Lipid films with a high resistance were obtained particularly on the ultra-flat surface of TSG used as the substrate/electrode. Hence it was concluded that the structure and morphology of the gold substrate play an essential role in the formation of tBLMs [13].

The formation of SAMs of DPTL on TSG slides was followed by SPR spectroscopy (see Fig. 2 and Ref. [14]). The thickness of the monolayer thus obtained is 4.5 ± 0.5 nm which, when compared with the length of the DPTL molecule in fully extended conformation (Fig. 1A), indicates a densely packed supramolecular array. The change in thickness during fusion of SAMs with DPhyPC liposomes (see inset of Fig. 2) shows an exponential increase, followed by a small linear increase which is most likely due just to adsorption of liposomes. The amplitude of the exponential part amounts to 3.7 nm, which corresponds to the value observed after washing. The resulting overall thickness of 8.2 ± 0.5 nm after liposome fusion is consistent with a SAM to which a monolayer of DPhyPC is added (see Fig. 1B for the dimension of DPhyPC), i.e. a lipid bilayer attached to the gold surface by means of tetraethyleneoxy spacers.

The electrical parameters of the lipid films were assessed by EIS and fitting the spectra to an equivalent circuit (Fig. 3). The resistances R_m obtained for different
Fig. 1. Structure and conformation of molecules used for the formation of tBLMs. (A) 2,3-di-O-phytanyl-sn-glycerol-1-tetraethylene glycol-DL-\(\alpha\)lipoic acid ester (DPTL) used to form SAMs. (B) DPhyPC used to prepare liposomes which were fused with SAMs. Conformations were obtained by molecular modelling using the program CS Chem 3D Pro.
TSG slides varied between 1 and 6 MΩ cm² for SAMs (lipid monolayer), and between 2 and 15 MΩ cm² for tBLMs (lipid bilayer). The relatively large ranges are thought to be due to differences in roughness of the TSG slides. In any case, the resistance of the monolayer was already quite high, indicating a high packing density of the supramolecular array. It increased upon vesicle fusion, as expected for the formation of a tethered lipid bilayer. The variation of the capacitances $C_m$ was less pronounced, hence average values of $1.2 \pm 0.4$ and $0.7 \pm 0.2$.

Fig. 2. SPR spectra of the bare TSG surface (squares), as well as of TSG coated with a DPTL monolayer before (circles) and after fusion with DPhyPC liposomes (triangles). The curves represent simulated spectra using the Fresnel equations [14] for a four-layer system of glass, gold, thiolipid and phospholipid. Refractive indices of 1.45 and 1.5 were assigned to the thiolipid and the phospholipid, respectively, while the refractive index of the glue was assumed to match that of the glass. The inset shows the change in thickness as a function of time during the fusion process of DPhyPC liposomes with a SAM. This change was followed at a constant angle of incidence ($55.5^\circ$) in the SPR spectrum.

Fig. 3. Electrical impedance spectra of the DPTL monolayer before (diamonds) and after vesicle fusion (triangles). The magnitude of the impedance, $Z$, and the phase angle, $\theta$, are represented by open and closed symbols, respectively. Excitation amplitude 10 mV, bias potential 0 vs. Ag | AgCl | NaCl(sat) reference electrode. The inset shows the equivalent circuit used for fitting the data. $R_m$ is the resistance of the bathing electrolyte solution, $R_m$ and $C_m$ are resistance and capacitance of the lipid membrane, respectively, while $C_m$ denotes a capacitance in the spacer region.
0.2 μF cm⁻² for monolayer and bilayer, respectively, can be calculated (the \(C_m\) value of the monolayer for the example listed in Table 1 is exceptionally low for unknown reasons). Capacitance and resistance of the tBLMs compare quite favorably to those of the freely suspended bilayer lipid membrane (BLM), which were reported as about 0.5 μF cm⁻² and \(\geq 10\) MΩ cm² [15]. Further characterizations, particularly concerning the roughness of the TSG surface as measured by AFM, as well as the fusion process which was followed by the quartz crystal microbalance technique and found to be fully consistent with a bilayer formation, will be given in a forthcoming publication. From the results obtained so far, the tBLM based on DPTL as the thiolipid can be considered as a good model system of the biological membrane.

### 3.2. Titration of valinomycin-doped tBLM with \(K^+\) ions

Valinomycin spontaneously partitions into a pre-formed lipid bilayer when an aliquot of an ethanolic stock solution is added to the aqueous bathing solution. The insertion of valinomycin into the membrane could be monitored by a decrease of the impedance as a function of time, which leveled off at about 300 kΩ cm² (not shown). Such a decrease is expected since valinomycin displays also a small \(Na^+\)-transport activity. However, a contribution from traces of \(K^+\) ions present cannot be excluded, although great care was taken to avoid any contamination. Aliquots of a KCl stock solution were then added stepwise to obtain different \(K^+\) concentrations starting in the micromolar range, and impedance spectra were recorded following every addition. From the results shown as Bode plots in Fig. 4 it is evident that the impedance decreases with increasing \(K^+\) concentration. It increased again when the \(K^+\) containing solution was replaced by the initial NaCl solution (not shown).

The EIS data in Fig. 4 were fitted to the conventional equivalent circuit [9,10] shown in Fig. 3, and the values of parameters thereby obtained are listed in Table 1. As expected, the membrane resistance (\(R_m\)) decreases with increasing \(K^+\) concentration, while the capacitance of the lipid layer (\(C_m\)) remains more or less constant. However, the capacitance \(C_m\) in the submembrane space varies considerably, which is difficult to understand in terms of current opinions. According to the analysis of Krishna et al. [16] performed with a similar system, this capacitance should consist of a constant Helmholtz capacitance \(C_h\) in series with a diffuse double layer capacitance \(C_d\). Krishna et al. found that \(C_h\) dominates the overall capacitance \(C_m\) for electrolyte solutions of higher ionic strength (\(>0.1\) M), whereas \(C_d\) becomes effective only in solutions of low ionic strength. While essentially \(Na^+\) ions permeated through the membrane due to gramicidin present in the system of Krishna et al., salt should permeate in our system due to the leak conductance, giving rise to the finite value of \(R_m\). In view of the long storage time of SAMs in NaCl solution and during liposome fusion it can be safely assumed that the salt is equilibrated between the bathing solution and the submembrane space (see also next section). Hence \(C_m\) should be more or less constant because of the high ionic strength in the bathing solution and the submembrane space. The minor increase in ionic strength due to the addition of KCl (cf. Table 2) can hardly explain the observed variations in \(C_m\). It thus becomes evident that the behavior of the valinomycin-mediated \(K^+\)-transport is most likely non-ohmic, and its representation by a resistor in an equivalent circuit should be used only as an approximation.

### 3.3. Network simulations with SPICE

SPICE is a computer program designed to simulate the behavior of electrical networks under different conditions. It includes programmable voltage and current sources which make it also possible to represent bioelectrochemical processes, such as the permeation of salt through a membrane or the valinomycin-
mediated K\(^+\) transport through a tBLM, in terms of electrical elements [11].

Salt permeation through a membrane is usually described by the integrated Nernst–Planck equation with the membrane capacitance \(C_m\) and the permeabilities \(P_i\) of the ionic species as parameters [11]. In the case of the DPTL SAM immersed in 0.1 M NaCl solution the permeabilities \(P_{Na} \approx 2 \times 10^{-10} \text{ cm s}^{-1}\) and \(P_{Cl} \approx 3 \times 10^{-10} \text{ cm s}^{-1}\) can be estimated from the leak resistance \(R_m = 5.3 \text{ M} \Omega \text{ cm}^2\) (Table 1) assuming that \(P_{Cl}/P_{Na}\) is about equal to the ratio of mobilities of these ions in solution. The volume of the spacer region per 1 cm\(^2\) of membrane area can be estimated as 2.2 nl by means of the length of 2.2 nm for the spacer molecule (Fig. 1A). A SPICE simulation with \(C_m = 0.59 \mu \text{F cm}^{-2}\) (Table 1), initially no salt in the spacer region, and a constant NaCl concentration of 0.1 M in the bathing solution then shows that a NaCl concentration of 0.09985 M is attained in the spacer region after 14 h of incubation, thus corroborating the assumption mentioned above.

Fig. 4. EIS data (Bode plots) of valinomycin-doped tBLM for different KCl concentrations in the bathing solution. The background electrolyte present in the bathing solution and the spacer region was 0.1 M NaCl, valinomycin concentration [val] = 4 \times 10^{-6} \text{ M}. KCl concentration in mmol l\(^{-1}\) and line type (from top to bottom) 0, solid; 0.02, dashed; 0.04, dotted; 0.09, solid; 0.19, dash-dot; 0.39, dash-dot-dot; 0.89, solid; 1.89, dashed; 6.89, dotted.
Table 2

<table>
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<tr>
<th>$10^3 [K^+]_{int}/M$</th>
<th>$10^6 [K^+]_{int}/M$</th>
<th>$\Delta \phi_{mem}/mV$</th>
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<td>0.04</td>
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<td>6.89</td>
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</table>

Parameter values used are $[\text{val}]_0 = 4 \ \mu M$, $C_m = 0.67 \ \mu F \ cm^{-2}$, $C_m = 3.4 \ \mu F \ cm^{-2}$, $R_{\text{cs}} = 63 \ \Omega \ cm^2$, $R_{\text{shock}} = 14.9 \ \text{M} \Omega \ cm^2$, $V_m = 2.2$ nl, temperature $32^\circ C$.

Stark et al. [12] could satisfactorily analyze experimental data obtained with the $K^+$/valinomycin system in negatively charged BLMs by means of the four-state kinetic scheme shown in Fig. 5A. Valinomycin (val) incorporated into the membrane can adopt two conformations (indicated by the subscripts 1 and 2) which are able to bind $K^+$ from and release it to the adjacent aqueous phases. In a symmetrical membrane the equilibrium constant for the transition between the two conformations has to be unity, i.e. the two rate constants for this transition are equal, as indicated by $k_t$ in Fig. 5A. For the same reason the binding rate constants $k_b$ as well as the dissociation rate constants $k_d$ of complex formation are the same for both conformations. The rate constants $k_{32}$ and $k_{23}$ for the transition between the two conformations of the charged $K^+$/val complex are sensitive to the electric field in the membrane which can be expressed as follows [11].

$$k_{32} = k_t \exp(F(\phi_1 - \phi_2)/(2RT)) \quad \text{and} \quad k_{23} = k_t \exp(-F(\phi_1 - \phi_2)/(2RT))$$

Here $\phi_1$ and $\phi_2$ denote the Galvani potentials in the aqueous phases, while $R$, $T$, and $F$ have their usual meaning. According to Stark et al. [12] the total mole number of valinomycin, $n_v$, in the membrane can be approximated by

$$n_v \approx d_m A_m \gamma_v [\text{val}]_0$$

where $d_m$ and $A_m$ are the thickness and the area of the membrane, respectively, $\gamma_v$ denotes the partition coefficient of valinomycin between the membrane and the aqueous phase, while $[\text{val}]_0$ is its concentration in the aqueous phase. Parameter values used in the simulations are those given in Ref. [12], $k_b = 3 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$; $k_d = 5 \times 10^4 \text{ s}^{-1}$; $k_t = 2 \times 10^4 \text{ s}^{-1}$; $\gamma_v = 6 \times 10^4$.

Stark et al. [12] have determined $k_b = 5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, which includes the increased $K^+$ concentration at the surface of the negatively charged membrane, for further explanation see text and Ref. [11].
and estimated the value given above for an uncharged membrane.

Fig. 5B shows the electrical network designed to simulate the kinetic scheme in Fig. 5A. The nodes numbered 1–4 represent the four states of valinomycin, and their potentials with respect to ground indicate the probabilities of these states. The K\(^+\) concentrations \(c_1\) and \(c_2\) in the two aqueous phases are applied as potentials to nodes 10 and 11, while the currents through the branches attached to these nodes represent the flows of K\(^+\) into and out of the membrane, respectively. The Galvani potentials \(\phi_1\) and \(\phi_2\) are applied as potentials to nodes 12 and 13, respectively, and the current through the branch between these nodes indicates the transfer of charge across the membrane due to the K\(^+\) transport. This network is designed as a so-called subcircuit, which can be easily plugged into circuits representing the experimental system (see below), and thus replaces the resistance representing the membrane permeability for ions in conventional equivalent circuits. The \textsc{spice} code of this subcircuit is given in Appendix A.

The circuit used to simulate the transient behavior of the system after addition of KCl and when subjected to a sinusoidal a.c. potential is depicted in Fig. 6. The values of \(C_m\), \(C_{in}\), \(R_{in}\), and \(R_{ex}\) determined for the tBLM before addition of valinomycin (Table 1) were assigned to the elements \(C\_mem\), \(C\_in\), \(R\_leak\), and \(R\_ex\), respectively, while the value 2.2 nl for the volume of the spacer region per 1 cm\(^2\) of membrane area, estimated as explained above, was assigned to the element \(C\_Vin\).

Simulations were performed with \textsc{win\textsc{spice}3} (version 1.03.06), i.e. the version of \textsc{spice} for Windows which is in the public domain. Fig. 7 presents, by way of example, the results for some parameters of a system with valinomycin in the presence of 6.89 mM KCl. After attaining a steady state the system was subjected to a sine wave excitation with a frequency of 1 Hz and an amplitude of 10 mV (Fig. 7C). It is evident that not only the resulting a.c. current (Fig. 7C) but also the oscillation of the K\(^+\) concentration in the spacer region (Fig. 7A) and those of the probabilities for the valinomycin states (Fig. 7B) are phase-shifted with respect to the excitation wave. In particular, the oscillations of the transmembrane potential and of the current through the membrane (Fig. 7D) are not in phase, which shows that the valinomycin-mediated K\(^+\) transport through the membrane cannot satisfactorily be represented by a resistance in an equivalent circuit.

In principle the impedance and the phase for the given frequency and KCl concentration can be calculated from the curves in Fig. 7C, but constructing a Bode plot with this procedure would be rather tedious. A much faster way to obtain such a plot is to use the a.c. small signal analysis mode of \textsc{spice}. However, there is a certain disadvantage of this approach in that \textsc{spice} ‘shortens capacitors’ in the d.c. analysis performed prior to the a.c. analysis. Hence, the capacitor representing the spacer volume in the circuit of Fig. 6 had to be replaced by a constant voltage source whose value was set equal to the K\(^+\) concentration reached at steady state after addition of KCl in a transient analysis. The corresponding values are listed in Table 2, together with the values of the membrane potential. The results of the a.c. simulations thus obtained are shown in Fig. 8 for all KCl concentrations, and together with the corresponding experimental data for a selected KCl concentration in Fig. 9.
Fig. 7. Simulation of a sine wave excitation of a tBLM in the presence of valinomycin and a constant KCl concentration of 6.89 mM in the bathing solution. (A) K⁺ concentration [K⁺]ₘ in the spacer region; (B) probability of states 1 (solid line) and 4 (broken line) in the kinetic scheme for valinomycin (Fig. 5A); (C) excitation potential Δφₑₓ (broken line) and resulting current density jₑₓ (solid line); (D) transmembrane potential Δφₘₚₙ (broken line) and current density jₘₚₙ through the membrane (solid line). Parameter values (as obtained from EIS data of the tBLM before addition of valinomycin): Cₘ = 0.67 μF cm⁻², Cₘₖ = 3.4 μF cm⁻², Rₑₓ = 63 Ω cm², Rₗₑₜₐₖ = 14.9 MΩ cm²; moreover Vₑᵣᵢₜ = 2.2 nl and temperature 32 °C. Sine wave excitation with 10 mV amplitude, 1 Hz frequency, and bias voltage 0.
4. Discussion and conclusions

It is evident from Figs. 4, 8 and 9 that the experimental data are reasonably well represented by the results of the simulations. It should be pointed out that none of the parameters in the simulations was adjusted, instead values obtained from the EIS data of the tBLM without valinomycin (Table 1) and from the analysis of Stark et al. [12] were used. Hence the agreement can be judged as even more satisfactory because the Bode plots were found to be rather sensitive to a variation of values for some parameters such as the rate constants of the
kinetic scheme in Fig. 5A. Moreover, the unrealistic variation of the capacitance $C_{in}$ in the spacer region, which emerged from the analysis by means of a conventional equivalent circuit (Table 1), is no longer necessary. Thus, both the suitability of tBLMs for quantitative studies of ion transport and the interpretation of the results by means of SPICE are demonstrated.

However, deviations between experimental and simulated data still exist, particularly in the low frequency domain of the phase plots. Possible reasons for this may be found in simplifications made in the SPICE simulations. In the a.c. simulations a constant value for the $K^+$ concentration in the spacer region had to be used, and the oscillations of this parameter observed in the sine wave simulations (cf. Fig. 7) are then eliminated which alters the frequency response of the $K^+$/valinomycin transport system. The small but finite $Na^+$ transport activity of valinomycin, which is liable to compete with the $K^+$ transport activity at least at low
K⁺ concentrations, was neglected. Moreover, the potential difference between the gold surface and the bathing solution was set to zero, in line with other authors who used similar systems [10]. This could be an impermissible approximation because a surface dipole potential at the metal|water interface due to the attachment of the spacer molecules is likely to exist [17] and the potential of the Ag | AgCl | NaCl(sat) electrode is disregarded. However, EIS using a Pt wire instead of Ag | AgCl | NaCl(sat) as the reference electrode yielded essentially the same spectra, which indicates that at least the potential of the reference electrode does not seem to be essential. Work directed towards a refinement of the SPICE simulations by taking these points into account is under way.

Acknowledgements

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Appendix A

SPICE code of the subcircuit called ‘valino’ as shown in Fig. 5B. For further information see Walz et al. [11], but note that these authors used SPICE 2G which does not yet include the programmable sources available in WINSPICE.

References